DEVELOPMENT AND FUNCTION OF NEUROSECRETORY SITES IN THE EYESTALKS OF LARVAL PALAEMONETES (DECAPODA: NATANTIA)[1]

JERRY H. HUBSCHMAN

U. S. Department of Health, Education, and Welfare, Public Health Service
Division of Water Supply and Pollution Control, Aquatic Biology Section
Robert A. Taft Sanitary Engineering Center, Cincinnati 26, Ohio

To comparative endocrinologists, *Palaemonetes* is a classic experimental animal. Five species inhabit the eastern United States. *Palaemonetes vulgaris* (Say), *P. pugio* Holthuis and *P. intermedius* Holthuis are marine or estuarine forms. *Palaemonetes kadiakensis* Rathbun and *P. paludosus* (Gibbes) inhabit fresh water. The larval development of all five species has been described (Broad, 1957; Broad and Hubschman, 1960, 1962; Faxon, 1879; and Dobkin, personal communication).

Our knowledge of the development of neurosecretory systems in larval crustaceans is meager and based essentially upon Pyle's (1943) work on *Homarus* and *Pinnotheres*. Matsumoto (1958) worked on newly hatched crabs; the species he studied, however (*Potamon dehaani*), like many fresh-water decapods, hatches in the form of a small adult, having passed within the egg those stages corresponding to the larval stages of other species. Dahl (1957) described the embryology of the X-organ in *Crangon allmanni*. His paper unfortunately contained no figures and no information on activity of the cells observed. Broch (1960) studied the effects of tissue extracts on isolated chromatophores of *Palaemonetes* zoeae. Costlow (1961, 1962) reported on the effects of *Sesarma* zoeal eyestalk extracts on adult chromatophores and the effects of removal on the megalops of *Callinectes*.

Experimental and histological data concerning the development of neurosecretory sites and functions in the eyestalks of larval *Palaemonetes* are presented here.

Methods

Rearing of larvae

The procedure for rearing was essentially the same for both fresh-water and marine species, and followed that outlined by Broad (1957). *Palaemonetes kadiakensis* was collected in and around the Sandusky Bay region of Western Lake Erie during the summer of 1960. This portion of the work was carried out at the Franz Theodore Stone Laboratory at Put-in-Bay, Ohio. The shrimp were kept in aquaria containing Lake Erie water and fed macerated parts of several local fishes, usually whole emerald shiner, *Notropis atherinoides*. Ovigerous females were examined daily. When embryos reached a late stage of development, the female was isolated in a culture dish until the eggs hatched. Upon hatching, the

[1] This work constitutes a portion of a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree from The Department of Zoology and Entomology of The Ohio State University.

entire brood was placed in a clean dish and fed day-old *Artemia* nauplii. The larvae were fed and the water changed daily until they reached a desired stage of development, at which time they were fixed. The larvae of some broods were fixed all at the same time, while from other broods, samples were removed throughout the developmental period.

Several females produced eggs in the laboratory. By using some of these eggs, a series of embryos was fixed for later sectioning. Eggs were fixed at two-hour intervals during the first day and 12-hour intervals thereafter until hatching. Individuals of one group were fixed at 6-hour intervals during the last three days of embryonic development.

Late embryos, larvae, and post-larvae of the other fresh-water species, *P. paludosus*, fixed in alcoholic Bouin's solution, were supplied by Mr. Sheldon Dobkin of the Marine Laboratory, University of Miami, Florida.

Rearing and experimental work on larval stages of the marine species were carried out at the Duke University Marine Laboratory, Beaufort, North Carolina. Adult *P. vulgaris*, *P. pugio*, and *P. intermedius* were collected in the tidal areas around Beaufort throughout the summer of 1961. The shrimp were maintained in sea-water aquaria on a diet of the soft parts of the mud snail, *Nassarius obsoletus*, the mussel, *Modiolus demissus*, and the oyster *Crassostrea virginica*. Females with eggs in late stages of development were isolated in individual bowls of sea water. After hatching, some larvae were reared in mass culture as above, while those used in experiments were placed in individual bowls (3″ Carolina stacking culture dishes), and complete molting and developmental histories were kept. All larvae were fed *Artemia* nauplii. The sea water in which larvae were reared was adjusted to 30 ± 1‰ salinity by the addition of demineralized water, and was changed every day. At the same time, the larvae were examined and a new supply of food was introduced.

There are five to eight molts prior to metamorphosis in *P. kadiakensis*, from six to eight in *P. intermedius*, and seven to eleven premetamorphic molts in *P. vulgaris* and *P. pugio* (Broad and Hubschman, 1962). To avoid confusion in this paper, all references to larval forms (Roman numerals) have been made in terms of the six described for *P. kadiakensis* (Broad and Hubschman, 1963).

Histological methods

All larval stages of *P. vulgaris*, *P. pugio*, *P. intermedius*, *P. kadiakensis*, and *P. paludosis* were sectioned. Preliminary to the study of larval tissues, the eyestalks of adult *P. vulgaris*, *P. pugio*, *P. intermedius* and *P. kadiakensis* were studied, establishing a basis for the interpretation of the developmental processes.

The fixing reagents used were 5% formalin, formalin-alcohol-acetic acid (FAA or AFA), Zenker's fluid, Helly's fluid, and Smith's alcoholic Bouin's solution (Guyer, 1953). Fixation time varied from 8 to 24 hours, depending upon the tissue and reagent. Separate eyestalks were washed in crustacean saline to remove blood and then immersed in alcoholic Bouin's solution. Freshly dissected eyestalks were studied by the methylene blue technique suggested by Bliss and Welsh (1952).

All tissues were sectioned by the paraffin method. Preparatory to dehydration and embedding, the larval cuticle was broken to insure penetration of the embedding medium. A tangential cut was made through the ommatidia at the distal end of the

eyestalk. Some thoracic appendages and the posterior portion of the abdomen were also removed. A similar tangential cut was made through the ommatidia of separate adult eyestalks. These operations were performed under a stereoscopic microscope at 40 ×.

Smith's alcoholic Bouin's fixative was found to produce the most satisfactory results. Problems of overfixation were not encountered, and the cuticle was not rendered brittle, as it was following Zenker or Helly fixation. The latter reagents also produced background coloration with the stains used. Fixation in formalin or AFA did not always provide good differentiation, but this was corrected by mordanting the sections in Bouin's fluid during the hydration process.

The most frequently used staining procedure was a modification of an azocarmine-aniline blue-orange-G process suggested by Gomori (1939, 1946). This modification has been described previously (Hubschman, 1962) and will be referred to from here on as azan.

Sections intended for general histological study were also stained in Mallory's triple connective tissue stain or with acid fuchsin followed by the counterstain of the azan process. Stains that are semiselective for neurosecretory material have been proposed by a number of workers. Two of these that have been widely used for crustacean tissues were employed here. The first, Gomori's (1941) chrome-hematoxylin phloxin, will be referred to hereafter as CHP. The other, Dawson's (1953) modification of Gomori's (1950) aldehyde fuchsin, will be designated PAF.

Destalking experiments

Experiments involving eyestalk removal from adult shrimp were primary in the sequence of events that originally established these structures as control centers of metabolic activity. To evaluate the role of eyestalks in larval development, a series of experiments was conducted with zoeal forms of *Palaemonetes pugio*. A group of sibling larvae (usually an entire brood) was separated into experimental and control groups. Each shrimp was placed in an individual bowl of sea water and assigned a number. Rearing methods were those described in the previous section. Complete molting and developmental records were kept for all larvae. Each day the shrimp were removed with a large-bore pipette and examined under a stereoscopic microscope. Any change in developmental form was recorded and the dish examined for the presence of exuviae.

Eyestalks were removed with a fine blade honed from a #2 insect pin mounted in a wooden handle. Three or four hours before the operation, the larvae were cooled to 10° C. The eyestalks were then severed at the base and the animals returned to room temperature over a period of two hours. Later, since it was found that the operation performed at room temperature resulted in no higher mortality than resulted after the cooling process, that step was eliminated. About 75% of the larvae survived the operation.

Two types of control animals were used. The first was unaltered, normal larvae. The antennae of the second type were removed to allow for possible differences in molting or development resulting from operative stress. Removal of the antennule, though an easy operation, resulted in excessive bleeding, owing to the relatively large size of the base of that appendage in early zoeal forms. Consequently, the antennal scale and flagellum of the operated control animals were removed by cutting

through the basal segment (either basipodite or coxopodite). Except for the operation, all larvae were treated in the same manner. The first zoea hatches from the egg with eyes enclosed in the protozoeal carapace and cannot be treated as a stalk-eyed form. Experiments were performed on all later larval stages.

Laboratory conditions did not permit precise control over temperature during the experiments. The room used was air-conditioned, but high outside temperatures and equipment operating in other parts of the laboratory placed a burden on the system. Thus, during the period of the experiments, the mean daily temperature fluctuated between 23.7° and 27.0° C. with an average of about 25.5° C. The average daily fluctuation in temperature was 3.47° C., but twice was 6.0° C.

Results

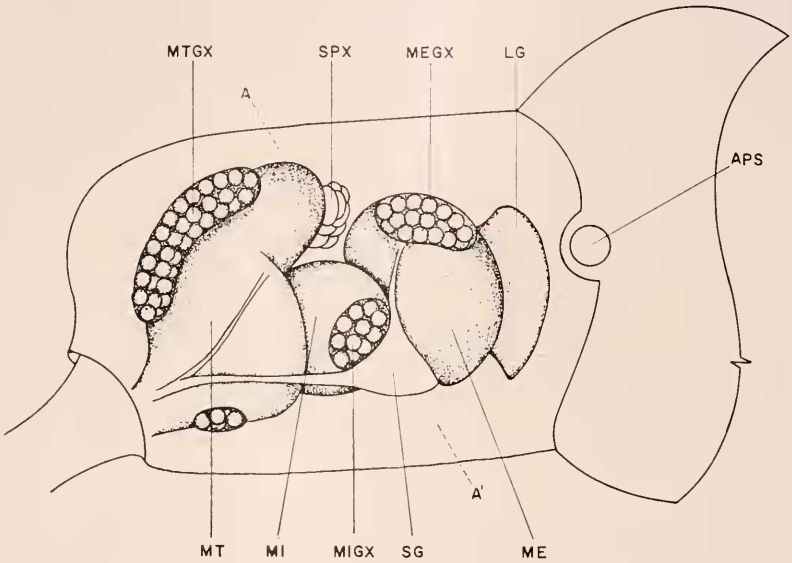
Adult eyestalks

As a basis for comparative study, eyestalks of adult shrimp were examined on the living animal, as freshly dissected eyestalks, and in serial histological section. The distribution of neurosecretory cell groups was found to agree closely with that described by previous workers. The eyestalk organs of *P. vulgaris* were pictured by Hanström (1939) in his survey of crustacean central nervous systems. The histology of the eyestalk of *Palaemonetes*, as well as that of *Uca*, was presented in some detail by Milburn (1958). An account of the anatomical aspects of neurosecretory systems in adult *Palaemonetes* and *Uca* is apparently in preparation at this time (Milburn, personal communication).

The eyestalks of *P. vulgaris*, *P. pugio*, *P. intermedius* and *P. kadiakensis* were found to be essentially alike. Figure 1 is a diagrammatic sketch of the eyestalk of *P. kadiakensis*. In keeping with the suggestion of Carlisle (1959), the terminology of Russell is used in describing the relationships of the structures discussed here. The sinus gland, shown to be composed of nerve fiber endings (Bliss and Welsh, 1952), is located on the dorsal, abaxial surface of the ganglionic mass, in a crevice between the *medulla interna* and the *medulla externa*. It surrounds the junction of the inner and outer blood sinuses. The X-organ of Hanström, the *pars distalis* or the sensory pore X-organ (referred to hereafter as SPX), occupies a position opposite the sinus gland (Fig. 2). It is located between the adaxial, ventral surface of the *medulla terminalis* and the cuticle. The sensory pore proper is positioned ventrally in the adult eyestalk. There are secretory cell groups associated with each of the ganglionic masses. Beginning at the proximal end of the eyestalk (Fig. 1) they are: the *pars ganglionaris* or *medulla terminalis* ganglionic X-organ (MTGX); the *medulla interna* ganglionic X-organ (MIGX); and the *medulla externa* ganglionic X-organ (MEGX).

Two important additional details were observed in fixed and stained preparations. The first was the existence of an extremely large, solitary, monopolar, secretory neuron. This cell is located on the abaxial surface of the *medulla interna* and closely associated with, but seemingly not contributory to, the sinus gland tract (Fig. 3). Its axon penetrates the fiber mass of the *medulla terminalis*. This giant neuron has a diameter of approximately 40 μ. It has a centrally placed nucleus (diameter 19 μ) containing two prominent nucleoli. The cell usually stains blue with azan and bronze to purple with PAF. This cell is an important feature of the larval eyestalk and will be discussed later.

The second histological feature that can be observed consistently is the vacuolar nature of the lobes of the SPX (Fig. 4). Hanström (1939) noted that the vacuolar portions appeared empty (except for secretory droplets) in preparations fixed in Bouin's solution, but they were filled with a stainable substance following Zenker fixation. (At times there were large accumulations of droplets in the vacuole



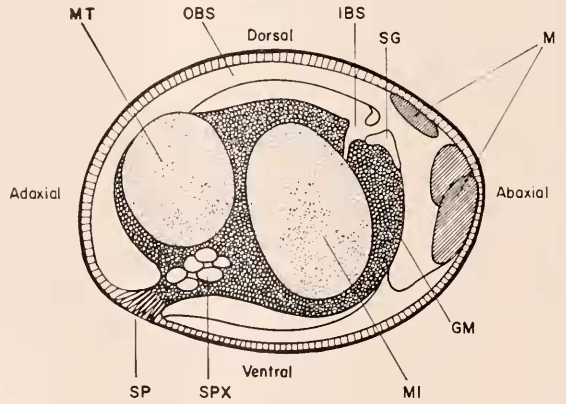


FIGURE 1. Dorsal view of the right eyestalk of adult *Palaemonetes kadiakensis*. The muscles, connective tissue, and most ganglionic nuclei have been omitted for clarity. APS, accessory pigment spot; LG, *lamina ganglionaris;* ME, *medulla externa;* MEGX, *medulla externa* ganglionic X-organ; MI, *medulla interna;* MIGX, *medulla interna* ganglionic X-organ; MT, *medulla terminalis;* MTGX, *medulla terminalis* ganglionic X-organ; SG, sinus gland; SPX, sensory pore X-organ.

FIGURE 2. Diagrammatic section through A-A′ of Figure 1. GM, nuclei of ganglionic mass; IBS, inner blood sinus; M, muscle; MI, *medulla interna;* MT, *medulla terminalis;* OBS, outer blood sinus; SG, sinus gland; SP, sensory pore; SPX, sensory pore X-organ.

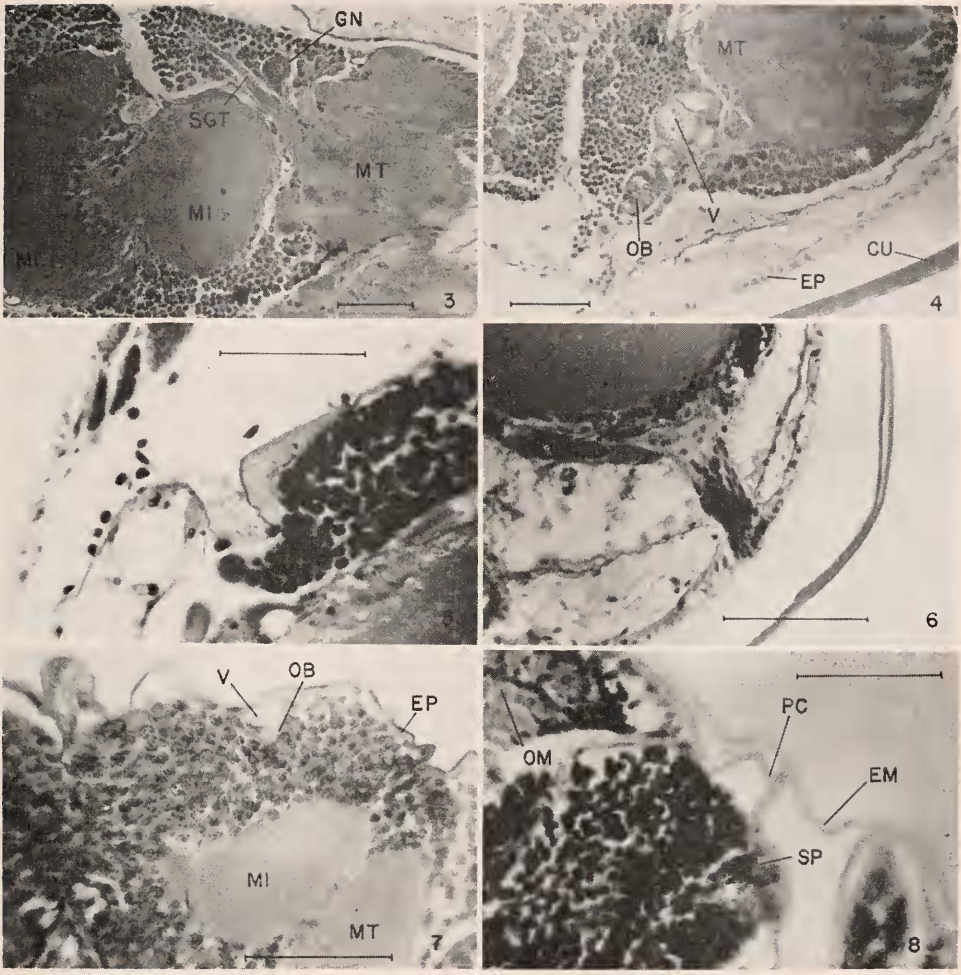


FIGURE 3. Frontal section through the eyestalk lobes of adult *Palaemonetes pugio* (azan stain). ME, *medulla externa;* MI, *medulla interna;* MT, *medulla terminalis;* GN, giant neuron; SGT, sinus gland tract. Scale represents 100 μ.

FIGURE 4. Section through sensory pore X-organ of adult *Palaemonetes pugio* (azan stain). CU, cuticle; EP, epidermal layer; MT, *medulla terminalis;* OB, onion bodies; V, vacuole with secretory droplets. Scale represents 100 μ.

FIGURE 5. Sinus gland of adult *Palaemonetes kadiakensis.* Compare with Figure 2 for anatomical relations (azan stain). Scale, 100 μ.

FIGURE 6. Sensory pore cells of adult *Palaemonetes kadiakensis.* Compare with Figure 2 (azan stain). Scale, 100 μ.

FIGURE 7. Section through eyestalk of *Palaemonetes kadiakensis* embryo (azan stain). EP, epidermal layer; MI, *medulla interna;* MT *medulla terminalis;* OB, *onion bodies;* V, vacuole of SPX. Scale, 100 μ.

FIGURE 8. Section through dorsal surface of embryonic eyestalk (azan stain). EM, egg membranes; PC, protozoeal cuticle; OM, developing ommatidia; SP, ganglionic cells elongating in the formation of the SPX. Scale, 100 μ.

regardless of fixation.) Hanström considered these structures to be secretory and attributed differences in appearance to stages in secretory production. Since then, the so-called onion bodies associated with these vacuoles have been shown to be nerve fiber terminations (Carlisle and Knowles, 1959).

Larval eyestalks

The histogenesis of neurosecretory structures was essentially the same in *P. kadiakensis, P. pugio, P. vulgaris* and *P. intermedius. Palaemonetes paludosus,* which has an abbreviated larval development (Dobkin, personal communication), passed through the same sequence of changes in a shorter period of time.

The incubation period of *P. kadiakensis* can be expected to range between 24 and 28 days (Broad and Hubschman, 1963). The topography of the definitive eyestalk is recognizable from about the tenth day. Prior to this, the eyestalk region is composed of undifferentiated neuroblasts. With differentiation, the nerve cells have characteristic brilliant red nuclei (following azan) and very sparse cytoplasm. The cytoplasm of these cells is confined almost entirely to the axons stretching far from the cell body. The neuropile appears pale purple. Undifferentiated neuroblasts contain cytoplasm that takes up the orange-G component of azan.

About 5 or 6 days before the hatching, the structures contributing to the future SPX can be seen. The most obvious feature of this complex is a single cavity or vacuole closely applied to the outer surface of the eyestalk (Fig. 7). This cavity contains a very fine filamentous substance, strands of which are near the limit of resolution of the light microscope. This has the appearance of connective tissue and stains blue with azan. The thickened wall of the cavity is in direct contact with the epidermal layer at this time. Onion bodies can be seen forming in nests of ganglionic cells. These are always more axial than the vacuole and do not contact the surface of the eyestalk. The onion bodies stain blue, but not as strongly as does the vacuolar portion. The vacuole-onion body complex (SPX) is surrounded by undifferentiated neuroblasts and occupies a position at the dorsal surface of the eyestalk. In some preparations a fine connection can be traced from the *medulla terminalis* to the SPX. It may be that a nerve fiber migrates some distance from the neuropile and then its terminal portion differentiates under the influence of specific cells. This gives the appearance of the nests mentioned earlier. It could not be determined whether or not the onion body was actually within the cytoplasm of the nest cell.

Just before the hatching, the onion bodies become more conspicuous. At this time they stain more deeply with aniline blue, but this may be simply because they are larger and more dense. Nerve cells on the surface of the ganglionic mass have begun to assume the elongated shape of sensory cells (Fig. 8). The sheath of the vacuole is now clearly cellular. Several onion bodies appear in the lower (axial) portion of the vacuole. At this time there seem to be three membranes present: one around the vacuole, one around the onion bodies, and an outer one covering both.

At hatching (Form I) the vacuole is approximately 15 μ in diameter. Individual onion bodies are up to 6 by 8 μ in diameter. Mitotic figures are numerous in the ganglionic mass. A presumptive neurosecretory cell appears in the crevice between the *medulla terminalis* and the *medulla interna* (Fig. 9). This is the

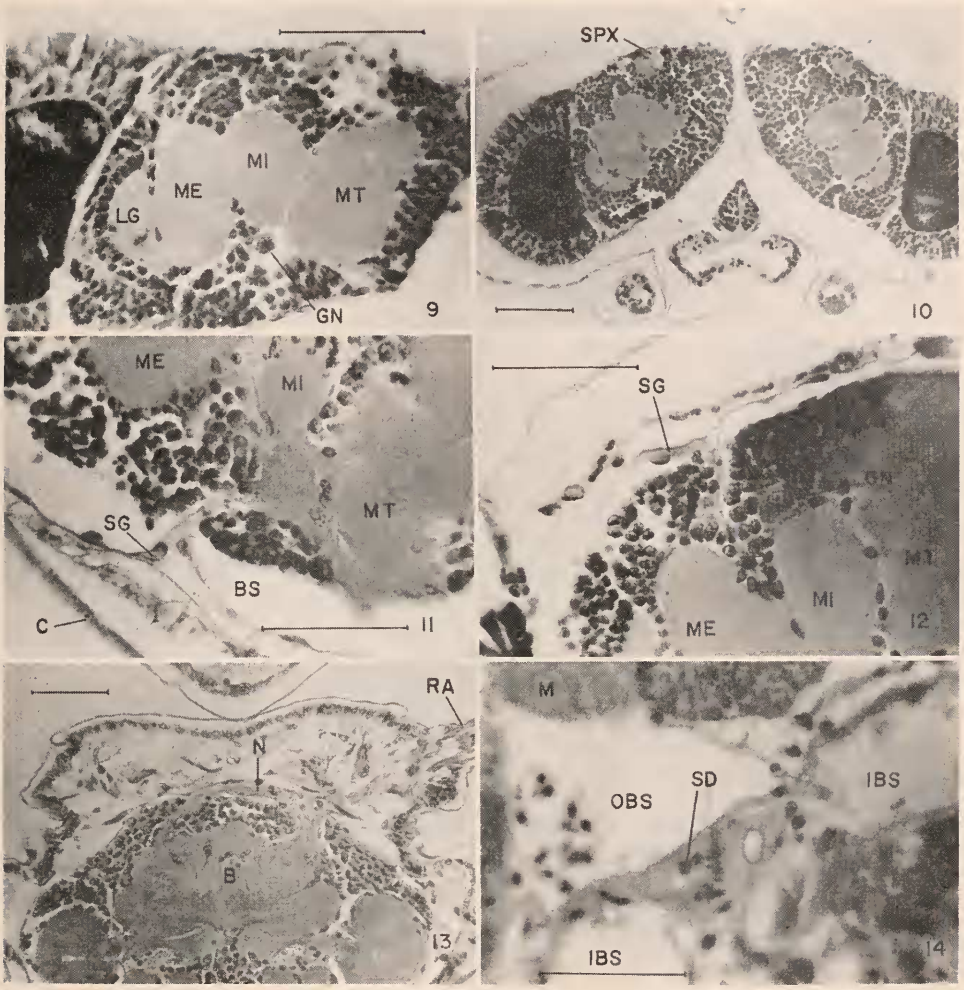


FIGURE 9. Section through the eyestalk of the first larval stage (Form I) of *Palaemonetes intermedius* (azan stain). GN, giant neuron in its primary (ventral) position; LG, *lamina ganglionaris;* ME, *medulla externa;* MI, *medulla interna;* MT, *medulla terminalis.* Scale, 100 μ.

FIGURE 10. Section through the anterior end of the first larval stage (Form I) of *Palaemonetes kadiakensis* (azan stain). SPX, sensory pore X-organ, (vacuole appears in eyestalk at left, onion body clusters can be seen in both eyestalks). Scale, 100 μ.

FIGURE 11. Sinus gland forming in the fifth larval stage (Form V) of *Palaemonetes pugio* (PAF stain). C, cuticle; BS, blood sinus; ME, *medulla externa;* MI, *medulla interna;* MT, *medulla terminalis;* SG, sinus gland. Scale, 100 μ.

FIGURE 12. Compact sinus gland of Form VII (first post-larva) of *Palaemonetes pugio* (PAF Stain). GN, giant neuron; ME, *medulla externa;* MI, *medulla interna;* MT, *medulla terminalis;* SG, sinus gland, 100 μ.

FIGURE 13. Section through the anterior end of *Palaemonetes pugio* from which eyestalks had been removed during larval development (azan stain). B, brain; N, nerve fiber tract crossing over and merging with nerve supply of regenerated appendage; RA, regenerated appendage. Scale, 100 μ.

FIGURE 14. Sinus gland of juvenile *Palaemonetes pugio* with accumulation of secretory material (azan stain). IBS, inner blood sinus; OBS, outer blood sinus; M, muscle; SD, secretory droplets; SG, sinus gland. Scale, 100 μ.

giant cell of the MIGX. It seems to differentiate from a ganglionic cell rather than from a typical neuroblast. This giant cell changes in staining reaction during the first larval stage. At the time of hatching it stains orange (as do the neuroblasts) but, by the first larval molt, the cell appears blue after azan and changes from pale to deep purple with PAF.

During the second larval stage (Form II), the onion bodies continue to increase in size and number. The SPX is by now a definite organ. It is no longer in contact with the surface of the eyestalk, but is still dorsal in position (Fig. 10). At this time, very small secretory droplets can be seen in the vacuole. These droplets are barely visible and appear only as dark specks after all stains.

By the time that the animal has reached Form III, the onion bodies become collectively larger than the vacuole. Individual bodies may range up to 11 by 16 μ in size, while the whole organ may be 45 μ in diameter. Neuroblasts around the

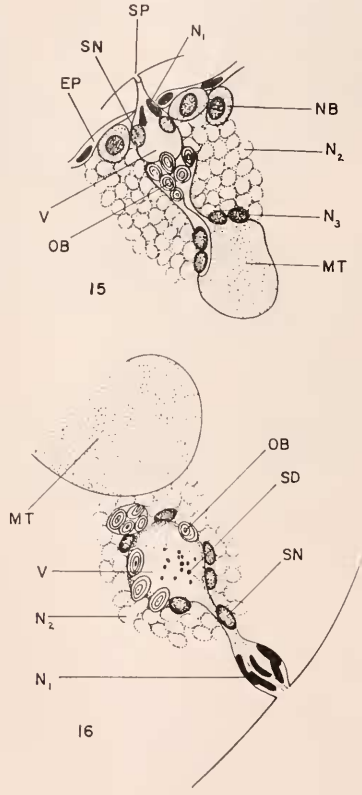


FIGURE 15. Diagram of sensory pore X-organ in the third larval form of *Palaemonetes*. EP, epidermal cell; MT, *medulla terminalis;* N_1, nucleus of sensory pore cell; N_2, nucleus of ganglion cell; N_3, nucleus of cell contributing a process to the SPX; OB, onion body; NB, neuroblast; SN, nucleus of sheath cell; SP, sensory pore; V, vacuole of SPX.

FIGURE 16. Diagram of sensory pore X-organ of post-larval *Palaemonetes*. MT, *medulla terminalis;* N_1, nucleus of sensory pore cell; N_2, nucleus of ganglionic cell; OB, onion body; SD, secretory droplet; SN, nucleus of sheath cell; V, vacuole.

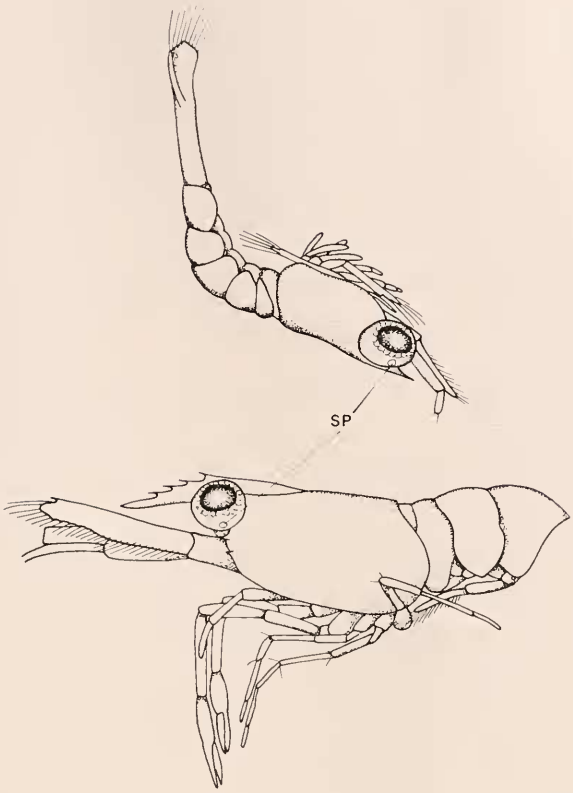


FIGURE 17. Diagram showing the attitude of larval (A) and post-larval (B) shrimp with reference to the relative position of the sensory pore X-organ.

SPX continue to divide. The vacuole is approximately 18 μ in diameter and contains more droplets. These droplets stain orange with azan and deep purple with PAF. A layer of ganglionic nuclei has now formed between the SPX and the surface of the eyestalk. Slightly elongated, epidermal, sensory cells project in a cone to the pore at the surface. The pore is approximately 5 μ wide and 7 μ deep (Fig. 15).

The SPX continues to be displaced, by differential growth, from its peripheral location to a site deeper in the ganglionic mass. Throughout larval life, the onion bodies increase in number while a single cavity remains.

Eventually, the onion bodies come to lie between the vacuole and the surface of the eyestalk, as they do in the adult. The accumulation of secretory droplets becomes continually greater. There is, however, no evidence of secretory activity by cells immediately surrounding the SPX.

By the fifth larval stage, but not earlier, the sinus gland can be recognized. While not a functional complex as yet, an accumulation of nerve endings can be seen at the site of the future junction of the external and internal blood sinuses (Fig. 11). The nerve fibers, mostly from the *medulla terminalis,* form a delicate

fan extending over the sinus area. The giant cell is in its definitive position and a number of presumptive neurosecretory cells occupy the site of the future MIGX. This area is ventrally located in the larval eyestalk.

The vacuole is a constant feature of the SPX complex from the fifth day before the hatch to the definitive adult organ. The adult conformation of the SPX can be recognized in the early post-larval shrimp (Fig. 16). At metamorphosis, the eyestalk begins to rotate on its longitudinal axis. The result is that, by the third post-larval molt, the SPX is ventral. This is just the opposite of the embryonic and larval position on the dorsal surface of the ganglionic mass. This rotation in effect results in the maintenance of the orientation of the sensory pore with the substrate, while the rest of the animal assumes the characteristic dorsal-side-up position of the adult (Fig. 17). In effect, the shrimp rotates around the fixed eyestalk.

At metamorphosis, the sinus gland develops its definitive shape (Fig. 12). No accumulation of secretory material could be detected during larval life. With the onset of post-larval existence (third or fourth post-larval molt), however, the sinus gland assumes functional importance. Examination of sections of destalked larvae revealed no build-up of stainable droplets in the brain stubs, as shown in adult crabs by Matsumoto (1958). Apparently the migration of neurosecretory substances from the brain and thoracic ganglion does not begin until post-larval life. The ganglionic X-organs of the adult eyestalks are not functional during larval development. Only after metamorphosis can the cells of the MTGX, MIGX, and MEGX be recognized histologically. With the procedures used, the secretory activity in these cells can be demonstrated for the first time in shrimp that have completed several post-larval molts.

Destalking experiment

In spite of the wealth of information available on decapod larval development, nothing is known of the mechanism of control of crustacean metamorphosis. Passano (1961) has suggested that an unknown hormonal control may exist, and has called our attention to the need for experiments involving interference with the normal process to support his hypothesis. The data reported here are derived from daily observations of 187 larval *P. pugio* (133 destalked larvae, 36 from which the antenna had been removed, and 18 intact controls). The mean intermolt period varies from 2.3 to 2.5 days, but the maximum duration of the intermolt does not exceed 5 days.

It seems obvious that the measurement of duration of molt cycle should have been made in some unit smaller than days. *Palaemonetes* larvae molt only at night, however (usually not before 8:00 or 9:00 PM), and observations more frequent than daily could add nothing of value to the data. The variate (*i.e.*, duration of molt cycle) is actually a discontinuous one and properly may be measured in days. No significant difference in duration of molt cycle could be observed between experimental and control animals.

Larvae from which eyestalks had been removed metamorphosed between 18 and 30 days after hatching (mean, 20.79 days). Operated control larvae metamorphosed between 20 and 25 days (mean, 22.88 days) and unaltered controls

metamorphosed between 18 and 22 days (mean, 20.33 days). Analysis of variance fails to reveal that these differences are significant.

Growth may properly be separated from development. It was convenient, however, to consider the influence of eyestalk ablation on growth of the same groups of larvae studied above. As shown by experiments on adult *Cambarus* (Smith, 1940), *Uca* (Abramowitz and Abramowitz, 1940), and *Carcinus* (Carlisle, 1955), destalking results in increased size. Destalked larvae at metamorphosis had an average overall length (tip of rostrum to end of telson) of 10.1 mm. Control animals were approximately 7.1 mm. at the same stage of development. No differences in average length could be observed between altered and intact control animals.

Adult shrimp differ from their brachyuran relatives in the chromatophore response following eyestalk removal. Crabs exhibit an overall paling while shrimp darken following the operation. In adult *Palaemonetes* there is complete dispersal of red pigments following removal of the eyestalks (Perkins, 1928). While quantitative aspects of these phenomena were not studied here, it should be noted that eyestalk removal resulted in complete paling of the larval shrimp. The pale condition persisted through subsequent molts and developmental phases through metamorphosis. The immediate condition seemed to result from contraction of chromatophores, but possibly was accompanied by loss of pigment in later stages.

It has been observed previously that removal of eyestalks proximal to the optic ganglia will result in regeneration of an antenna in place of the missing eyestalk, in some adult decapods (Bliss, 1960). Regeneration of a short, setose appendage was commonly observed among the larvae destalked in these experiments. No external eyestalk or portion thereof was ever observed to regenerate. Larvae having antennae removed were found to replace this structure quite rapidly. Often the full antenna was present by the time of metamorphosis. In several instances the nerve supply to the regenerated antennule (in eyestalk position) arose from both sides of the brain. This crossing-over is shown in Figure 13.

Fingerman and Aoto (1960) have demonstrated in *Cambarellus* that, after severance of eyestalks, an accumulation of secretory products can be seen in the terminal portion of the remaining stub. This supports the findings of Matsumoto (1958) mentioned earlier. Adult shrimp (*P. kadiakensis*) were destalked to see if this build-up could be observed. The animals were held for 6 days, then fixed, sectioned, and stained with azan. An accumulation of red droplets was found in the nerve stubs of the destalked shrimp. The optic nerves of intact adults were free of droplets. It should be recalled that no accumulation of stainable material could be detected in the brain stubs of destalked larvae.

Discussion

Dahl (1957), in his account of the embryology of the X-organs in *Crangon allmanni*, states that the first onion body lies directly beneath the integument and is surrounded by cells of the SPX. This is not the case in *Palaemonetes*. The most prominent feature of the SPX in this shrimp is the sac-like vacuolar portion, which, indeed, does contact the epidermal layer in early development (Fig. 7). The onion bodies are always more axial (*i.e.*, proximal to the neuropile) in position and never contact the integument. During larval development, however, these

structures do change in relative position when the onion bodies bypass the vacuole, owing to differential growth. By the time the adult stage is reached, the vacuole lies between the onion body cluster and the neuropile (Fig. 4). While the vacuole may not be present in *Crangon*, this difference could be explained on the basis of similarity of staining properties of these structures. The developing vacuole and associated onion bodies both appear blue after azan, purple after PAF, and gray after CHP. A tangential section of the vacuole near the surface of the eyestalk could be misinterpreted as an onion body.

This sac-like SPX in the larval eyestalk appears more like the X-organ of the Mysidacea than it does the definitive form of adult *Palaemonetes*. In the mysids, *Eucopia* and *Boreomysis*, the X-organ is sac-like and situated close to the eye papilla (Hanström, 1939). In one (*Boreomysis*), the large vesicle is filled with intensely stained, winding threads that call to mind the filamentous inclusions of the larval SPX in *Palaemonetes*. The resemblance of many decapod larvae to the Mysidacea is superficial and probably reflects the pelagic environment (Snodgrass, 1956), but it is interesting that there is internal similarity, at least in one detail.

Other aspects of the developing SPX in *Palaemonetes* agree more closely with that in *Crangon*. Cells of the SPX, as well as the ganglionic X-organs, develop from neuroblasts of the ganglionic layer. As is the case in *Crangon*, these neuroblasts first assume the appearance of regular nerve cells, then mature into definitive types. Many of the cells of the SPX differentiate during embryonic development, and those of the ganglionic X-organs become recognizable only after metamorphosis. The first onion body appears 5 or 6 days before the hatch. As Dahl (1957) observed, this coincides with the appearance of eye pigments. There is no known significance to this occurrence, other than that it furnishes an outward clue to the state of development. This could be of aid to the experimental embryologist.

Carlisle (1953) has shown that onion bodies in *Lysmata* are expanded endings of nerve fibers emerging from the *medulla terminalis*. This appears to be the case in *Palaemonetes*. A fine connection can be traced from the *medulla terminalis* to the developing onion body. The end of this fiber appears to differentiate into an onion body while in close association with cells of the ganglionic mass. The developing body is surrounded by nuclei that seem to participate in the maturation process. This gives the appearance of the rosette or nest mentioned earlier. Since the nerve tract connecting the onion body cluster with the neuropile is not visible until the onion bodies form, the direction of growth cannot be stated with certainty. There is no reason, however, to believe that the nest cells send out the process in the direction of the *medulla terminalis*.

The remainder of the SPX, including the terminal sensory pore portion, is derived from cells of the nerve ganglion. There is no evidence to support the view that the sensory pore complex is derived from epidermal cells. Late in embryonic development, the sensory portion develops from ganglion cells that elongate within the ganglionic mass (Fig. 8).

Throughout embryonic development and larval life, the cells of the future ganglionic X-organs fail to show any activity with CHP or PAF. The single exception to this is the giant cell located peripherally to the MIGX, close to the sinus gland tract. This cell apparently becomes active early in larval life. During the first phase of free-living existence (Form I), this cell stains deeply

with aniline blue and appears purple with PAF (Fig. 9). Since this is the only cell that seems to be active during larval life, and its activity slightly precedes the accumulation of droplets in the SPX, it may well be the source of this stainable material. Admittedly, the CHP and PAF techniques are selective rather than specific for neurosecretion (Bliss, Durand and Welsh, 1954), but previous work has shown physiological activity to be associated with the products of cells demonstrated with these processes (Passano, 1951a, 1951b, 1954). Carlisle (1954) has reported the movement of droplets from the ganglionic X-organs to the SPX. He suggests that the SPX is a point of storage and release for neurosecretory products. This is probably true of *Palaemonetes*. Since there was no accumulation of stainable material at the stubs of the brain in destalked larvae, the droplets found in the vacuole of the SPX probably were produced within the eyestalk. In adult eyestalks (Fig. 3), the axon of the giant cell can be seen to enter the neuropile of the *medulla terminalis* and is not directly incorporated into the sinus gland tract. The fibers could not be traced out of the neuropile.

Pyle (1943) described the position of the X-organ (SPX) in embryos of *Pinnotheres* and *Homarus*, as what will become the median ventral side of the eyestalk in the first zoea. This is very different from what I found in *Palaemonetes*. The SPX develops embryologically in that portion of the eyestalk that becomes the dorsal surface in the free-swimming larva. It remains dorsal throughout larval life, becoming ventrally located during the changes associated with metamorphosis. At metamorphosis, the eyestalk rotates on its longitudinal axis, the dorsal surface becomes ventral and the ventral, dorsal. All internal structures retain a constant relation to each other. It is interesting that shrimp zoeae swim upside down and backwards during larval life. At metamorphosis, the animal assumes the dorsal-side-up position of the adult. The sensory pore remains oriented toward the substrate throughout this change in attitude (Fig. 17). A functional difference between dorsal and ventral surfaces of the eyestalk was hinted at by Keeble and Gamble (1904), but their experiments involving changes in direction of illumination failed to support this idea. From the structural complexity of the SPX, especially in the adult shrimp, it is difficult to believe that the so-called sensory pore does not have functional importance.

The sinus gland cannot be seen in *Palaemonetes* until the fifth larval stage (Form V). Between this and final stage (Form VI), the nerve fiber endings comprising the sinus gland become increasingly compact. Staining reactions indicate that the structure does not become functional until post-larval life. Pyle (1943) found that the sinus gland was not recognizable until the third stage after hatching in *Homarus americanus*. By the fourth stage, the sinus gland had increased in size, but still failed to show the brilliant stain of the adult structure. Morphologically, the fourth stage in *Homarus* is a post-larva, in spite of its continued pelagic existence (Herrick, 1895). The activity of the sinus gland (*i.e.*, storage and release of neurosecretory products), seems to be limited therefore, to post-larval life. After metamorphosis, the internal development can no longer be related to external morphology. In *Palaemonetes*, the sinus gland appears to be functional after the second or third post-larval molt (Fig. 14). Because of difficulties with their rearing, Pyle was not able to study the development of *Pinnotheres* larvae. He does state, however, that the sinus gland was not evident in late embryos or in the first zoeal stage.

Broch (1960) reported that all larval stages of *P. vulgaris,* from hatching through the "8th zoeal stage," responded to background coloration with changes in chromatophores. He found that the chromatophores completely contracted over a white background and were fully expanded over a dark background. He stated that the red chromatophore, on an excised thoracic segment of a second zoea, contracted upon the addition of brain extract, expanded upon the addition of abdominal nerve cord extract, and contracted when eyestalk extract was added. It was not stated that the tissue extracts were of adult origin. Broch concluded (p. 306) that "endocrine control of chromatophores in the zoea of *P. vulgaris* is comparable to that of the adult."

Perkins (1928) showed that eyestalk removal resulted in dispersal of red chromatophores in adult *Palaemonetes.* Preliminary experiments with *P. pugio* larvae indicate that the response to eyestalk removal in larvae is opposite to that in the adult. Destalked larvae became pale following the operation; the pale condition persisted through metamorphosis. This reaction, opposite or otherwise, indicates that there is some influence exerted on the chromatophore system by larval eyestalks.

Costlow (1961) has reported finding a *Uca* black-dispersing substance in the eyestalk extracts obtained from zoeae of *Sesarma reticulatum.* The chromatophore-dispersing substance fluctuates in total activity during the molt cycle. Recognizing the possibility of different hormones controlling color change and molting, Costlow (p. 184) suggests the following hypotheses:

"(1) The *Uca* black-dispersing substance located within the eyestalks of the larvae fluctuates because of changes in titre associated with moulting but is not directly responsible for, nor involved in, the actual moulting process, or (2) if the endocrine organs within the eyestalks of the larval stages are responsible for production or storage of several hormones, including the moult-inhibiting hormone, the reduction of the titre of the *Uca* black-dispersing substance may reflect the reduction in titre of the moult-inhibiting hormone also" *(sic).*

Results of the destalking experiment described above suggest that chromatophores and molting are under separate control in the larvae of *Palaemonetes.* Eyestalk removal definitely affected the chromatophores (contracted) of the larvae, while the operation seemed to have no influence on the frequency of molting.

The molt-inhibiting hormone has been related to the X-organ-sinus gland complex in all decapods studied (Carlisle and Knowles, 1959). In light of the historical evidence for lack of activity in the ganglionic X-organs, and the absence of a functional sinus gland during larval life, the failure of eyestalk loss to interfere with molting frequency does not seem unreasonable. If a molt-inhibiting hormone is present in larval shrimp, it is probably produced somewhere other than in the eyestalks. Possibly the molting cycle in the larva goes uninhibited. The rapid cycle (two-day intermolt) may go on continually as a function of the production of molting hormone by a larval molting gland, if one exists. The duration of proecdysis (Drach's stage D) in adult natantians represents 60+% of the molt cycle (Passano, 1960). Morphological details of new spine formation suggest that the proecdysal period may be almost continuous, or at least of longer relative duration, during larval development (Broad and Hubschman, 1963).

The accumulation of droplets in the larval SPX may represent the storage and/or

release of a chromatophorotropic hormone. The deprivation of such a substance (or one component) results in the overall paling in larval shrimp. A similar material in the eyestalks of *Sesarma* larvae may be the *Uca* black-dispersing substance demonstrated by Costlow. It is noteworthy that *Sesarma* appears to be unique among crabs since it darkens in response to eyestalk removal (Enami, 1951; Prosser and Brown, 1961). This behavior is like that of adult *Palaemonetes* and opposite to the reaction in all other brachyurans. Carlisle (1955) has shown that molt inhibition and control of water balance are effected by separate hormones in *Carcinus*. If the relatively large size of destalked larvae is a result of increased water uptake, the control of these processes would seem to be separate in *Palaemonetes* larvae as well. The destalked larvae at metamorphosis were approximately 50% larger than the control animals. There was no difference in the duration of the intermolt periods between these groups. This suggests another possible factor attributable to the secretory products in the SPX.

This work was supported in part by National Science Foundation Summer and Cooperative Fellowships. This aid is gratefully acknowledged.

I am indebted to Mr. Ron Engel of The Ohio State University for his assistance in making field collections. Mr. Sheldon Dobkin, of the Marine Laboratory, University of Miami, made an important contribution by supplying specimens of *Palaemonetes paludosus*. Very special thanks are due Dr. A. Carter Broad, under whose guidance this work was carried out.

Summary

1. The histogenesis of eyestalk organs in *Palaemonetes vulgaris*, *P. pugio*, *P. intermedius*, and *P. kadiakensis* is essentially the same. *Palaemonetes paludosus*, having an abbreviated larval development, follows the same sequence of events in a shorter period of time.

2. The developing eyestalk of *Palaemonetes* has two consistent, characteristic features: (a) a single, large, monopolar, secretory neuron that becomes active during the first larval stage, and (b) a sac-like vacuole in the sensory pore X-organ that appears to be the site of storage and release of secretory products, from the first larval stage through adult life.

3. The sensory pore X-organ develops from neuroblasts of the eyestalk ganglia. It appears 5 or 6 days before hatching. It is located dorsally and remains in this position throughout larval development. Secretory droplets appear in the SPX vacuole during the first larval stage. The accumulation of droplets increases throughout larval life.

4. The sinus gland is not recognizable until the fifth larval stage. It apparently does not function as a neurohemal organ until after metamorphosis.

5. At metamorphosis, the eyestalk rotates on its longitudinal axis. The dorsal surface becomes ventral and the ventral, dorsal. Since the animal turns over at this time, the orientation of the sensory pore remains constant with relation to the substrate.

6. Eyestalk removal had no effect on the larval molting cycle, nor on metamorphosis.

7. Eyestalk removal results in complete paling of larval *Palaemonetes*. The pale condition persists throughout larval development and metamorphosis.

8. There is no accumulation of secretory material in the brainstubs of destalked larvae. It is concluded that the droplets found in the SPX originate within the eyestalk.

9. It is suggested that the giant neuron is the source of stainable material found in the larval SPX.

10. The ganglionic X-organs are not functional during larval development.

11. The endocrine systems operating in *Palaemonetes* larvae differ in several respects from those of the adult.

LITERATURE CITED

Abramowitz, R. K., and A. A. Abramowitz, 1940. Moulting, growth, and survival after eyestalk removal in *Uca pugilator*. *Biol. Bull.*, **78**: 179–188.

Bliss, D. E., 1960. Autotomy and Regeneration. *In:* The Physiology of Crustacea. Vol. 1, T. H. Waterman, ed. Academic Press, New York.

Bliss, D. E., J. B. Durand and J. H. Welsh, 1954. Neurosecretory systems in decapod Crustacea. *Zeitschr. Zellf. mikr. Anat.*, **39**: 520–536.

Bliss, D. E., and J. H. Welsh, 1952. The neurosecretory system of brachyuran Crustacea. *Biol. Bull.*, **103**: 157–169.

Broad, A. C., 1957. Larval development of *Palaemonetes pugio* Holthuis. *Biol. Bull.*, **112**: 144–161.

Broad, A. C., and J. H. Hubschman, 1960 The larval development of *Palaemonetes kadiakensis* Rathbun. *Anat. Rec.*, **137**: 343–344.

Broad, A. C., and J. H. Hubschman, 1962. A comparison of larvae and larval development of species of Eastern U. S. *Palaemonetes* with special reference to the development of *Palaemonetes intermedius* Holthuis. *Amer. Zool.*, **2**: 394–395.

Broad, A. C., and J. H. Hubschman, 1963. The larval development of *Palaemonetes kadiakensis* Rathbun in the laboratory. *Trans. Amer. Micr. Soc.* (in press).

Broch, E. S., 1960. Endocrine control of the chromatophores of the zoeae of the prawn *Palaemonetes vulgaris*. *Biol. Bull.*, **119**: 305–306.

Carlisle, D. B., 1953. Studies on *Lysmata seticaudata* Risso (Crustaceae: Decapoda). VI. Notes on the structure of the neurosecretory system of the eyestalk. *Pubbl. Staz. Zool. Napoli*, **24**: 435–447.

Carlisle, D. B., 1954. The X-organ sinus gland complex, somatotropin, the ovarian inhibiting hormone and sex reversal in *Lysmata* (Natantia: Hippolytidae). *Pubbl. Staz. Zool. Napoli*, **24** (suppl.) : 79–80.

Carlisle, D. B., 1955. On the hormonal control of water balance in *Carcinus*. *Pubbl. Staz. Zool. Napoli*, **27**: 227–231.

Carlisle, D. B., 1959. On the sexual biology of *Pandalus borealis* (Crustacea: Decapoda). I. Histology of incretory elements. *J. Mar. Biol. Assoc.*, **38**: 381–394.

Carlisle, D. B., and F. G. W. Knowles, 1959. Endocrine Control in Crustacea. Cambridge Monographs in Experimental Biology. Cambridge Univ. Press.

Costlow, J. D., Jr., 1961. Fluctuations in hormone activity in Brachyura larvae. *Nature*, **192**: 183–184.

Costlow, J. D., Jr., 1962. The effect of eyestalk extripation on metamorphosis of megalops of the crab *Callinectes sapidus* Rathbun. *Amer. Zool.*, **2**: 401–402.

Dahl, E., 1957. Embryology of the X-organs in *Crangon allmanni*. *Nature*, **179**: 482.

Dawson, A. B., 1953. Evidence for the termination of neurosecretory fibers within the pars intermedia of the hypophysis of the frog *Rana pipiens*. *Anat. Rec.*, **115**: 63–69.

Enami, M., 1951. The sources and activities of two chromatophorotropic hormones in crabs of the genus *Sesarma*. I. Experimental analyses. *Biol. Bull.*, **100**: 28–43.

Faxon, W. A., 1879. On the development of *Palaemonetes vulgaris*. *Bull. Mus. Comp. Zool. Harvard*, **5**: 303–330.

FINGERMAN, H., AND T. AOTO, 1960. Effects of eyestalk ablation upon neurosecretion in the supraesophageal ganglia of the dwarf crayfish, *Cambarellus shufeldti*. *Trans. Amer. Micr. Soc.*, **79**: 68–74.

GOMORI, G., 1939. Studies on the cells of the pancreatic islets. *Anat. Rec.*, **74**: 439–460.

GOMORI, G., 1941. Observations with differential stains on human islets of Langerhans. *Amer. J. Path.*, **17**: 395–406.

GOMORI, G., 1946. Staining of chromaffin tissue. *Amer. J. Clin. Path. (Tech. Suppl.)*, **10**: 115–117.

GOMORI, G., 1950. Aldehyde fuchsin: a new stain for elastic tissue. *Amer. J. Clin. Path.*, **20**: 665–666.

GUYER, M. F., 1953. Animal Micrology. University of Chicago Press, Chicago, Illinois.

HANSTRÖM, B., 1939. Hormones in Invertebrates. Oxford University Press.

HERRICK, F. H., 1895. The American lobster: a study of its habits and development. *U. S. Fish. Comm. Bull. for 1895, pp. 1–252.*

HUBSCHMAN, J. H., 1962. A simplified azan process well suited for crustacean tissue. *Stain Tech.*, **37**: 379–380.

KEEBLE, F., AND F. W. GAMBLE, 1904. The colour physiology of higher Crustacea. *Phil. Trans. Roy. Soc. London, Ser. B*, **196**: 295–389.

MATSUMOTO, K., 1958. Morphological studies on the neurosecretion in crabs. *Biol. J. Okayama Univ. (Japan)*, **4**: 103–176.

MILBURN, N. S., 1958. Neurosecretion in crustaceans. Ph.D. Thesis, Harvard University (Radcliffe College).

PASSANO, L. M., 1951a. The X-organ sinus gland neurosecretory systems in crabs. *Anat. Rec.*, **111**: 502.

PASSANO, L. M., 1951b. The X-organs, a neurosecretory gland controlling molting in crabs. *Anat. Rec.*, **111**: 559.

PASSANO, L. M., 1954. Phase microscopic observations of the neurosecretory product of the crustacean X-organ. *Pubbl. Staz. Zool. Napoli*, **24**: 72–73.

PASSANO, L. M., 1960. Molting and its control. *In:* The Physiology of Crustacea. Vol. I, T. H. Waterman ed. Academic Press, New York.

PASSANO, L. M., 1961. The regulation of crustacean metamorphosis. *Amer. Zool.*, **1**: 89–95.

PERKINS, E. B., 1928. Color changes in crustaceans, especially *Palaemonetes*. *J. Exp. Zool.*, **50**: 71–105.

PROSSER, C. L., AND F. A. BROWN, JR., 1961. Comparative Animal Physiology. W. B. Saunders Co., Philadelphia.

PYLE, R. W., 1943. The histogenesis and cyclic phenomena of the sinus gland and X-organ in Crustacea. *Biol. Bull.*, **85**: 87–102.

SMITH, R. I., 1940. Studies on the effects of eyestalk removal upon young crayfish (*Cambarus clarkii* Girard). *Biol. Bull.*, **70**: 145–152.

SNODGRASS, R. E., 1956. Crustacean metamorphosis. *Smithsonian Misc. Coll., 131, No. 10.*